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PRINCIPAL INVESTIGATOR: Richard H. Finnell, Ph.D.

CONTRACTING ORGANIZATION:
Texas A&M Research Foundation
College Station, TX 77845

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Richard H. Finnell, Ph.D.

Email: rfinnell@ibt.tamhsc.edu

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7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
Texas A&M Research Foundation
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2121 W. Holcombe Blvd
Houston, TX 77030

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14. ABSTRACT
Perturbed folate levels are a possible risk factor for autism: alterations in methionine metabolism in autistic patients may be due to a functional folate deficiency, and folate receptor autoimmunity has been linked to cerebral folate deficiency and autism. Supplemental folate has proven to be an effective treatment in these individuals. Mouse models of altered intracellular folate transport and metabolism exist (Folr1, Folr2, Mthfr, and PCFT1). We hypothesized that folate deficiency secondary to genetic manipulation will cause both decreased CSF 5MTHF and altered (autistic-like) behavioral phenotypes in mice, and that supplemental folate will remediate the phenotypes. Folr2 nulls display a deficiency in nest building that is ameliorated by folate supplementation. Pcft1 hets displayed significantly increased dominance behaviors in the tube test; this tendency was not ameliorated by folate supplementation. Ablation of genes in the folate pathway may result in abnormal adult behavior. This utility of folate in restoring normal behavior differs in a consistent pattern based on the exact point at which the folate pathway is disrupted.

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INTRODUCTION

Intracellular folate transport and metabolism are key for proper embryonic development and perturbed folate levels have been suggested as a possible risk factor for autism(*1*). Metabolic and genetic data support the idea that alterations in methionine metabolism found in autistic patients may be due to a functional folate deficiency (*2*). Recently, folate receptor autoimmunity has also been linked to both cerebral folate deficiency and autism (*3*). Further, supplemental folate has been shown to be therapeutic in these individuals and others (*4*). Several genetic mouse models of altered intracellular folate transport and metabolism are in existence (Folr1, Folr2, Mthfr, and PCFT1). These models have been previously utilized to evaluate the role of folate in birth defects such as neural tube and conotruncal heart defects (*5*). Despite the evidence that folate transport and metabolism plays a prominent role in some forms of autism, to date, none of these mouse models have been utilized to assess neurobehavioral development. Casual observation of these mice suggests such deficits. Hence, we propose a novel application of these unique animal models to evaluate their utility in autism research.

We hypothesize that folate deficiency secondary to genetic manipulation will cause both decreased CSF 5MTHF and altered (autistic-like) behavioral phenotypes in mice, and that supplemental folate will remediate the phenotypes. Collectively, this work will assess 4 unique murine models of folate deficiency for their utility in autism research, and provide the foundation for new avenues of research into the role of folate and folate supplementation in autism.

BODY

In order to assess the effects of folate deficiency in mice, we evaluated four different mouse models of altered folate transport or metabolism for both CSF folate levels and adult behavior based on a small battery of assays designed to characterize a behavioral phenotype. We also determined the efficacy of treatment of these models with supplemental folate. We utilized the already existing genetically modified Folr1, Folr2, and Pcft knock out mice to severely limit folate transport into the cell. We also utilized the genetically modified Mthfr knock out mouse as a model of altered folate metabolism.

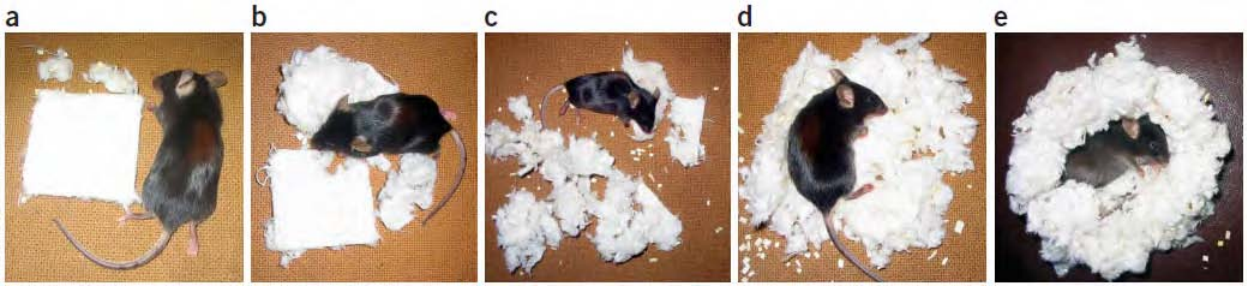
Animals and Housing: Mice were housed in the Institute of Biosciences and Technology Vivarium, which is fully accredited by the Association for Assessment and Accreditation of Laboratory Animal Care. The animals were maintained in clear polycarbonate microisolator cages and were allowed free access to food and water (Harlan Teklad Rodent Diet #8606, Ralston Purina, St. Louis MO). The mice were maintained on a 12-h light/dark cycle. Nulligravid females, 50–70 days of age, were mated overnight with males and examined for the presence of vaginal plugs the following morning, and the onset of gestation was considered to be 10 p.m. of the previous night, the midpoint of the dark cycle. All experiments were performed on gravid dams.

This work involved 4 independent mouse models of folate deficiency: Folr1, Folr2, Pcft1, and Mthfr genetically modified mice. Heterozygous breeding pairs were crossed to generate offspring of 3 different genotypes: +/+, +/-, and -/-. As not all genotypes produce viable offspring without folate supplementation, this study was limited to those genotypes that survive in the non-supplemented state.

- Pcft1: Wildtype (WT) and heterozygous (het) offspring were compared. Folate supplementation of additional heterozygotes was achieved though weekly IP injection of s-folinic acid (SFA) (20 mg/kg) to dams throughout pregnancy and lactation and to offspring following weaning.
- Folr2: WT and nullizygous (null) offspring were compared. Folate supplementation of additional nulls was achieved through administration of a high folate diet (HFD) (Dyets) (40 mg/kg) to dams throughout pregnancy and lactation, and to offspring following weaning.
- Mthfr: WT and het offspring were compared. Folate supplementation of additional hets was achieved through administration of betaine in drinking water (2%) to dams throughout pregnancy and lactation, and to offspring following weaning.
- Folr1: WT and het offspring were compared. Folate supplementation of additional hets was achieved through administration of a high folate diet (HFD) (Dyets) (40 mg/kg) to dams throughout pregnancy and lactation, and to offspring following weaning.

Behavioral Assays and Statistical Analysis: All treatment groups were evaluated between the 12 and 20 weeks of age using 4 different neurobehavioral assays: nest building to begin to assess behavioral deficits; marble burying for perseverative/impulsive behaviors; 3-chambered box for social interaction, and tube test for social dominance. Each assay was performed sequentially such that the least stressful assay was performed first, and the most stressful performed last: nest building, marble burying, 3-chambered box for social interaction, and tube test. All knockout animals were compared to WT using appropriate statistical analysis, as described below. Because of the increased incidence of autism in males, male mice were used exclusively in these studies.

- Nesting: Animals were housed in cages of sibling with mixed genotype. Upon initial separation in to individual cages, mice were tested for their ability to build nests. Three grams of Nestlet were placed in each cage each cage 1 hour prior to the onset of the dark cycle and left undisturbed overnight. Nest-building was assessed based on a 5-point scale. (1- Nestlet not noticeably touched; 2- Nestlet partially torn; 3-Nestlet mostly shredded, but no identifiable nest; 4 - identifiable, but flat nest; and 5- a (near perfect nest.) Data were analyzed using a 2-tailed Mann-Whitney analysis.



(a–e) These nests are assigned scores of 1–5, respectively. An anesthetized mouse has been used here because of the difficulties in obtaining a satisfactory image with a freely moving mouse (image from (*6*)).

- Marble burying: Mice were tested for marble burying as an index for perseverative/impulsive behavior. Mice are presented with 16 marbles in a 4 X4 grid placed lightly on a gently tamped volume of aspen chip bedding 5 cm deep, and allowed 30 minutes to bury marbles. As mice dig, marbles are incidentally submerged in litter. Marbles are considered buried if >70 of the marble volume is submerged. The number of marbles buried is recorded and analyzed using a 2-tailed Mann-Whitney analysis.

- The three-chambered social testing apparatus was designed at NIH in order to measure various aspects of sociability in mice. Three chambers are linked by close able gates; tansitions between chambers and time spent in each chamber may be recorded by a computerized monitoring program. We explored the preference of a mouse to investigate, and spend time with, a novel stranger mouse as opposed to a novel object, which would be a normal preference.

  The test mouse was placed in the closed central chamber (2) of the apparatus, and allowed to acclimate for 10 minutes. Next, a novel stranger mouse was placed under an upside wire pencil cup in chamber 1 or 3, with an empty upside-down wire pencil holder placed in the remaining chamber, the gates between the chambers were raised, and recording initiated. Total seconds spent with the novel stranger versus the novel object was analyzed by a 2-tailed paired Mann-Whitney test. Failure to achieve a statistically significant preference for spending time with a novel stranger was considered indicative of abnormal behavior.

  A set of novel stranger mice had been previously acclimated to being restrained for 10-minute periods under the pencil cups. The stranger mice were alternated from side 1 to side 3 in order to determine that a preference for a specific side by the test mice did not confound the outcome. Stranger

mice were age-matched C57Bl/6J, and were not used more than 4 times each. The apparatus was housed in a room with an ambient noise level of 55 db, and placed directly under a light. The apparatus was wiped down with 70% ethanol at the onset of investigations, and between mice thereafter.

- The tube test for social dominance determines a preference, when challenged with a novel stranger mouse at opposing ends of a 12" length of PVC pipe, to yield to the novel stranger and back out of the tube (lose) or complete transit of the tube while the novel stranger yields was (win). Stranger mice were age-matched C57Bl/6J. Each test mouse was challenged 3 times with a novel stranger mouse (a match). Mice are placed headfirst at opposite ends of a tube and released simultaneously. The match ends when one retreats from the tube and is assigned a score of 0 (lose); the remaining mouse is assigned a score of 1 (win). Stranger mice were generally used 3 times, and no more than . Both test mice and stranger mice were separately acclimated to the testing apparatus in advance of the trials. Failure to achieve a win/loss out come in 4 minutes, or the mice crossing over each other to exit opposite ends of the tube, resulted in the match not being scored. The outcome (win=1, loss=2) was recorded, and analyzed using a 2-tailed Fisher's Exact test.
  Results

CSF Collection: After all behavioral assays have been completed, a terminal collection of folate in the CSF is performed. CSF is isolated from the cisterna magna compartment. Mice are anesthetized with pentobarbital (75 mg/kg i.p). The animal is then placed on a narrow platform beneath a dissecting microscope. The tissue above the cistern magna is excised with care not to puncture the translucent meninges. The surrounding area is gently cleaned with the use of cotton swabs to remove any residual blood or other interstitial fluid. A sterile insulin needle is used to puncture the arachnoid membrane covering the cistern. The CSF, which is under positive pressure as a result of blood pressure and respiration, begins to flow out of the needle entry site once the needle is removed. A pulled glass pipette is used to collect CSF as it exits the compartment. Once the needle is completely removed, the pipette is lowered onto the puncture site and used to remove any remaining CSF. The primary collection usually takes less than 15 s. The cistern will refill with several microliters of CSF within several minutes. A second collection is then performed to increase the net yield. Concentrations of folate are currently being determined by immunohistochemistry and will be reported upon completion of these assays.

Results

Nest Buulding Assay: Folr2 heterozygotes displayed a strongly statistically significant tendency toward poorer quality nests, with large pieces (1/3 to 1/2) of the Nestlet material left untouched. Normal nest building behavior was restored in the folate-treated nulls. No other animals displayed statistically significant deficieccies in nestbuilding.

Representative nest built by Folr2 WT with mouse

Representative nest built by Folr2 null

Group A	Group Size	Mean	SEM	p-value
Pcft1 WT	n=6	5	0	--------
Pcft1 Het	n=7	5	0	--------
Pcft1 Het+ SFA	n=9	5	0	--------

Group B	Group Size	Mean	SEM	p-value
Folr2 WT	n=9	4.7	0.2205	--------
Folr2 Null	**n=6**	**2.7**	**0.6146**	***0.0212**
Folr2 Null + HFD	n=6	4.3	0.6667	0.7609

Group C	Group Size	Mean	SEM	p-value
Mthfr WT	n=8	4.5	0.5	--------
Mthfr Het	n=6	5	0	--------
Mthfr Het + Betaine	n=6	4.5	0.5	0.5

Group D	Group Size	Mean	SEM	p-value
Folr1 WT	n=5	4.8	0.2	--------
Folr1 Het	n=8	4.9	0.0625	0.8154
Folr1 Het+ HFD	n=5	5	0	----------

Tables 1A-1D: Nestlet Assay: Data were analyzed using the 2-tailed Mann-Whitney Test. "*" denotes statistical significance.

Marble-Burying Test: There were no statistically significant differences in marble burying behavior when genetically modified mice were compared to WT.

Group A	Group Size	Mean	SEM	p-value
Pcft1 WT	n=6	14	0.3651	----------
Pcft1 Het	n=7	12.9	1.654	0.7173
Pcft1 Het+ SFA	n=9	12.3	0.7817	0.1729

Group B	Group Size	Mean	SEM	p-value
Folr2 WT	n=9	10.4	1.435	----------
Folr2 Null	n=6	10.2	2.151	0.906
Folr2 Null + HFD	n=6	10.3	2.028	0.9061

Group C	Group Size	Mean	SEM	p-value
Mthfr WT	n=8	11	1.524	----------
Mthfr Het	n=6	14.2	0.7032	0.136
Mthfr Het + Betaine	n=6	12.7	0.5578	0.9483

Group D	Group Size	Mean	SEM	p-value
Folr1 WT	n=5	13	0.4472	----------
Folr1 Het	n=8	12.8	1.221	0.6587
Folr1 Het + HFD	n=5	13.2	0.9695	0.8334

Tables 2A-2D: Marble Burying Test: Data were analyzed using the 2-tailed Mann-Whitney Test. "*" denotes statistical significance.

Three-Chambered Testing Box for Social Interaction: All genotypes and treatments were evaluated separately to determine if they spent more mean time with a novel stranger than with a novel object, which is indicative of normal social interaction. **Note that failure to achieve statistical significance indicates a lack of preference to spend time with a novel stranger, which is abnormal**. By this measure, nearly all animals in this study displayed abnormal behavior: only folate-treated Pcft1 het and Folr2 nulls displayed normal behavior. Further evaluation of this data will be performed and reported.

Group A	Group Size	Mean time with Stranger (sec)	Mean time in empty chamber (sec)	p-value
Pcft1 WT	n=6	285	202	**0.2938**
Pcft1 Het	n=7	379	138	**0.0217**
Pcft1 Het+ SFA	n=9	374	137	*0.0028

Group B	Group Size	Mean time with Stranger (sec)	Mean time in empty chamber (sec)	p-value
Folr2 WT	n=9	280	160	**0.2714**
Folr2 Null	n=6	307	146	**0.1523**
Folr2 Null+ HFD	n=6	416	65	*0.0008

Group C	Group Size	Mean time with Stranger (sec)	Mean time in empty chamber (sec)	**p-value**
Mthfr WT	n=8	229	211	**0.8252**
Mthfr Het	n=6	226	181	**0.4605**
Mthfr Het+ Betaine	n=6	299	183	**0.2043**

Group D	Group Size	Mean time with Stranger (sec)	Mean time in empty chamber (sec)	p-value
Folr1 WT	n=5	235	240	**0.9785**
Folr1 Het	n=8	237	148	**0.2993**
Folr1 Het+ HFD	n=5	267	267	**0.3733**

Tables 3A- 3D: 3-Chambered Testing Appar atus for S ocial In teraction: ea ch genotype was evaluated separately. All data were analyzed using 2-tailed paired Mann-Whitney test. * p<0.05.

Tube Test f or Social Dominance: Both Pcft1 hets displayed statistically significant increases in wins when compared to WTs that was not restored to control levels in SFA-treated mice. This may indicate a difficulty in understanding social int eraction cues, or increased aggression. SFA-tre ated mice display a ma rginally lower win rate, but interestingly, 22 % of t he matches were not co mpleted by the 4 minute time limit, and thus were not scored. Similarly, this may indicate a difficu lty understanding social interaction cues, as the high number of unscored matches indicates that they are not simply deferring to the control animal.

Group A	Trials	Wins (%)	SEM	p-value
Pcft1 WT	n=18	33.3	0.1143	----------
Pcft1 Het	**n=20**	**85**	**0.08192**	***0.0023**
Pcft1 Het + SFA	n=21	71.4	0.101	[a]*0.021

Group B	Trials	Wins (%)	SEM	p-value
Folr2 WT	n=27	44.4	0.09745	----------
Folr2 Het	n=17	23.5	0.106	0.2076
Folr2 Het + HFD	n=16	47.1	0.1291	1

Group C	Trials	Wins (%)	SEM	p-value
Mthfr WT	n=21	28.6	0.101	----------
Mthfr Het	n=21	14.3	0.07825	0.4537
Mthfr Het + Betaine	n=18	27.8	0.1086	1

Group D	Trials	Wins (%)	SEM	p-value
Folr1 WT	n=14	50	0.1387	----------
Folr1 Het	n=19	78.9	0.09609	[b]0.1361
Folr1 Het+ HFD n=1	5	80	0.1069	0.1281

Tables 3A-3D: Tube Test for Social Dominance. All data analyzed using the 2-tailed Fischer's Exact test. "*" denotes statistical significance.
[a]22% of matches 'timed out', leaving the match unscored.
[b]21% of matches"timed out", leaving the match unscored.

Key Research Accomplishments

Folate knockout mice have been evaluated for their adult behavior. CSF has been collected, and is currently being analyzed by immunohistochemistry to determine if CSF folate levels are associated with observed behavioral abnormalities.

Reportable Outcomes

Folr2 nulls display a deficiency in nest building that is ameliorated by pre- and postnatal folate supplementation. Pcft1 hets displayed significantly increased dominance (wins) in the tube test in comparison to WT; this tendency was not ameliorated by pre- and postnatal folate supplementation. All groups displayed abnormal behavior in the 3-chambered testing apparatus for social interaction that remains to be characterized more fully.

Conclusion

Ablation of genes in the folate pathway may result in abnormal adult behavior. This utility of folate in restoring normal behavior differs in a consistent patter n based on the exact point at which the folate pathway is disrupted.

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